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Determination of the Absolute Configuration and the Conformation of Carbohydrate Molecules Based on the Approach of Analytical Organic Chemistry

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Summary

Conformation and absolute configuration are the main factors to define the three dimensional structures of carbohydrate molecules. In this study, analytical tools of nuclear magnetic resonance spectroscopy (NMR) and high performance liquid chromatograpy (HPLC) were coupled with new chemical manipulations to yield new analytical methods based on analytical organic chemistry. This allowed us to unambiguously determine both the absolute configuration and conformation of sugars. For the conformational analyses, stereoselective deuteration was coupled with NMR methods, while for the configurational analysis, a new fluorescent chiral derivatizing agent, (S)-TBMB carboxylic acid was developed and combined with the HPLC analysis. The backgrounds, the results and the applications of these new methods were reviewed.

With the rapid increase of interests in biological roles of glycoproteins and glycolipids on cell surfaces, the development of practical methods to determine the stereochemistry of carbohydrate molecules have become an important research objective. Owing to the recent developments of high resolutional nuclear magnetic resonance spectroscopy (NMR) and related computer technique, precise information of the molecular structures of oligosaccharides has become available. These methods based on spectroscopy or theoretical calculations, however, can not solve all problems in the complicated stereochemistries of carbohydrate molecules. In our continuous efforts to determine the three dimensional structures of carbohydrate molecules and the relating bio-organic compounds, we have taken a common approach incorporating chemical methods into the spectroscopic methods like circular dichroism (CD), optical rotatory dispersion (ORD), NMR and high performance liquid chromatography (HPLC).

In this paper, three parts are reviewed from our stereochemical studies of

carbohydrate molecules. They are as follows:

(i) *Conformational analysis based on chiral deuteration and NMR spectroscopy.*

(ii) *Highly sensitive analysis to determine the D, L-configurations based on the fluorescent chiral derivatization and HPLC.*

(iii) *Discovery of the first regiomistaken reaction of UDP-galactosyl transferase.*

In the first section (i), chiral deuteration as a chemical method was coupled with NMR methods to determine the conformational preferences of 1-6 linked saccharides, while in the second one (ii) a new fluorescent chiral derivatizing agent (S)-TBMB carboxylic acid was developed and applied to the HPLC analysis. In the third section, the stereochemical study was extended to the enzymatic reactions, galactose oxidase, lipases and UDP-galactosyl transferase (GalT).

(i) Conformational Analysis Based on Chiral Deuteration and NMR Spectroscopy

Three dimensional structures of oligosaccharides can be defined by two angles around the linkage (ϕ, φ, Figure 1). For the 1, 6-linked oligosaccharides, another rotational angle (ω) around the C5-C6 bond (hydroxymethyl group) exists and makes the conformational analysis more difficult. For the conformational analyses of oligosaccharides, NMR spectroscopy has provided the most powerful tools since the hetero- (C-H) or homogeneous (H-H) coupling constants (J value) and the nuclear overhauser effects (NOE) give direct information on the conformational property around the observed atoms (C, H). Although optical rotation ($[\alpha]_D$) had long been used for the conformational analysis, its use has

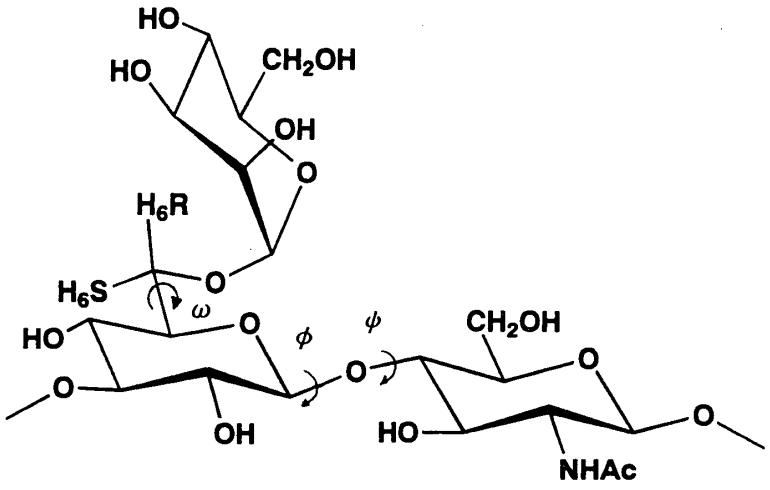


FIG. 1. Three kinds of dihedral angles (ϕ, φ, ω) to define the three dimensional structure of oligosaccharides and the definition of H-6proR (H_6R) and H-6proS (H_6S) protons.

been replaced by the NMR method and limited to the assignments of the linkage and the anomeric configurations or to the assignment of the D, L-configurations (see the second section). In addition to these spectroscopic methods, various sorts of empirical or theoretical energy calculations have been applied in which HSEA program, developed for the conformational analyses of carbohydrate molecules, has been most widely employed (1-5). Based on these methods described above, the analyses of 1-2, 1-3 and 1-4 linked saccharides have become possible in a consistent manner. On the other hand, the conformational analysis of the 1-6 linkage bearing the rotation of the hydroxymethyl group was still difficult, and therefore the results based on the NMR, optical rotation and energy calculations gave inconsistent results even for the analysis of monosaccharides. The main reason for the difficulty of the conformational analysis of the 1-6 linkage point by the NMR spectroscopy was clear ; the discrimination of the two prochiral protons at C-6, *i.e.*, H-6proR and H-6proS (Figure 1) was difficult even though they gave separate ^{1}H-signals. Since each of their the vicinal coupling constants with H-5 ($J_{H5,H6S}$, $J_{H5,H6R}$), chemical shifts and NOE can inform the conformational property, the unambiguous discrimination of the two C-6 protons was the key for the conformational analysis by ^{1}H-NMR.

In order to solve this problem, selective deuteration at C-6 will provide the most effective way since the ^{1}H-signal replaced by a deuterium disappears in the spectrum. This approach had been already attempted by several groups (6-8), and conformations of per-*O*-acetylated monosaccharides had been successfully

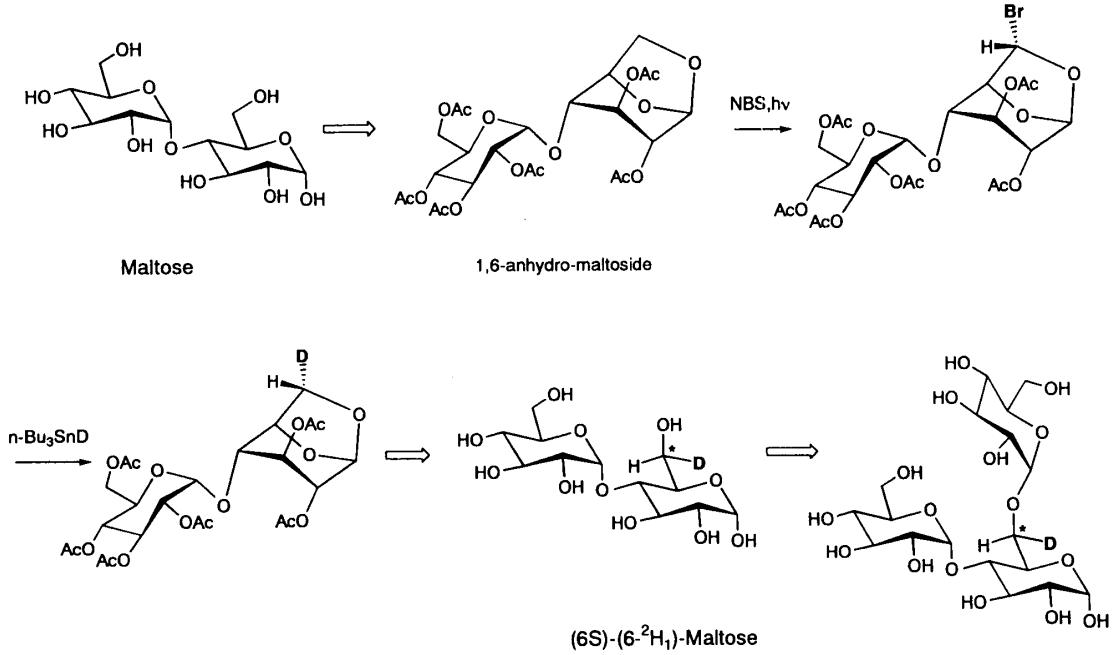


FIG. 2. Synthetic pathways towards chirally deuterated maltose and branching trisaccharide of amylopectin type (*lit.* 23).

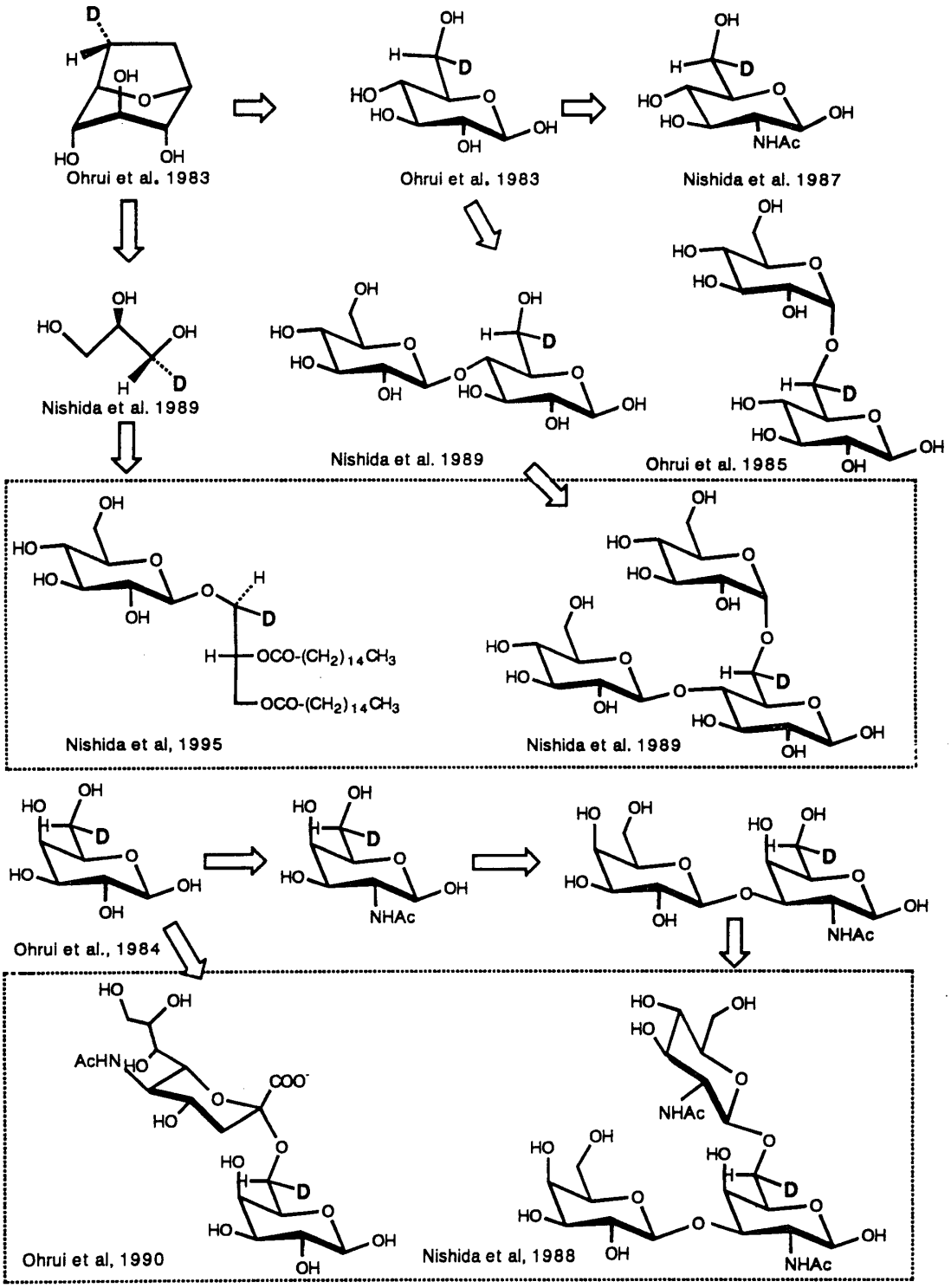


FIG. 3. Some examples of deuterated sugars and the relating compounds prepared for the conformational studies.

determined. However, application of this approach could not be extended to free sugars nor to oligosaccharides probably because of the low resolution of the NMR machine at that time. Another reason may be the low stereoselectivity of the deuteride reductions. More recently, Kakinuma (9-10) reported highly selective methods to prepare chirally deuterated D-glucoses [(6S)- and (6R)-(6-$^2H_1$)-D-glucoses] and applied them to the biosynthetic studies of antibiotics. Application of these synthetic methods to the other sugars for the conformational studies, however, seemed to be difficult.

Soon after Kakinuma reported his method to prepare chirally deuterated D-glucoses, Ohrui, in our group, reported a more convenient method to prepare chirally deuterated D-glucoses *via* a photobromination of 2, 3, 4-tri-*O*-benzoyl 1, 6-anydro-β-D-glucose [Ferrier reaction (11)] and the deuteride reduction with tributyltin deuteride (12-14). Extention of this approach to D-galactose could be performed straightforwardly (15). The high stereo selectivity as well as the ease of the chemical procedures prompted us to prepare a series of chirally deuterated monosaccharides, 1-6 linked disaccharides, trisaccharides and the relating organic compounds as the models for the conformational analyses (Figures 2 and 3).

By using these chirally deuterated sugars for the NMR analysis (16-19), the two prochiral protons, H-6proR and H-6proS could be unambiguously

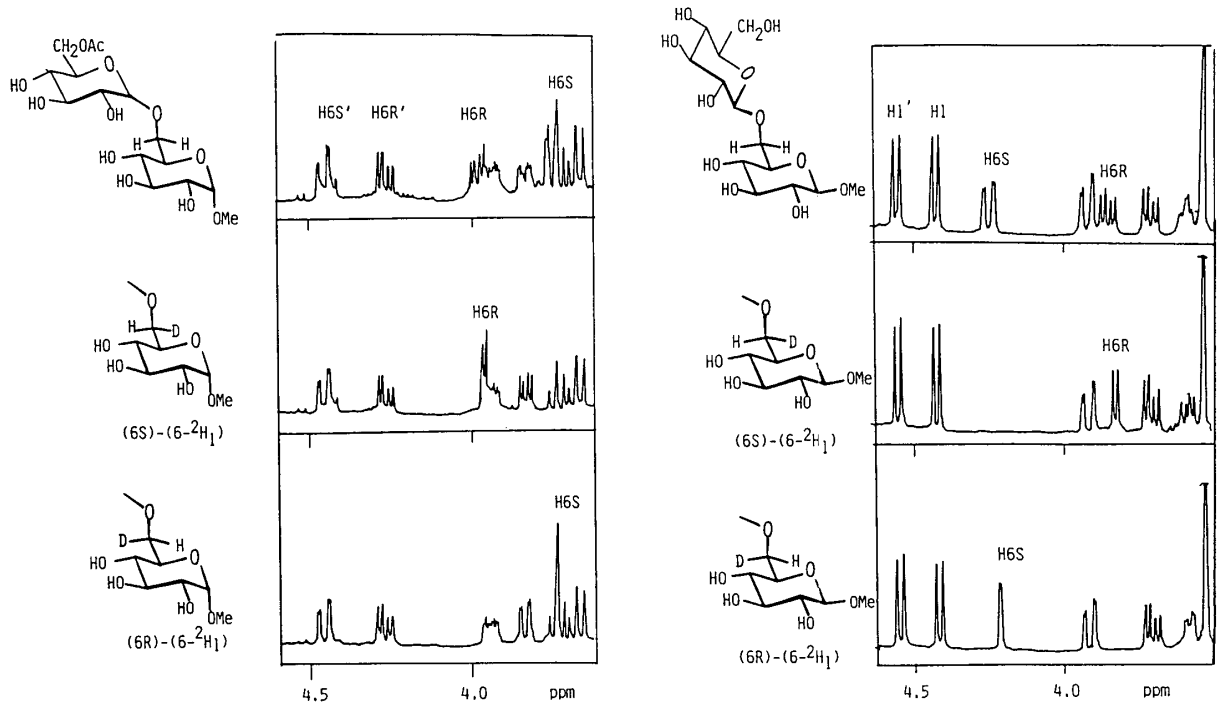


FIG. 4. ^{1}H-NMR spectra of chirally deuterated methyl α-isomaltosides (left) and methyl β-gentiobiosides (right) in D_2O (*lit.* 20).

differentiated from each other through the disappearance of the signal by the deuteration (Figure 4). Moreover, the vicinal coupling constants of each of prochiral H-6 protons with H-5 gave direct information to calculate the rotamer distribution of the three conformers, namely *gg*, *gt* and *tg*, around the C5-C6 bond (Figure 5). For the calculation, we have proposed new equations based on Karplus theory taking into account possible deviations of dihedral angles (19). These studies, based on the chiral deuteration and the NMR analysis, showed that the conformational preference of the hydroxymethyl group was largely different between D-glucoses and D-galactoses with a different configuration at the C-4 position, and the change of the configuration at C-1, C-2 and C-3 showed a much smaller influence on the conformation. For D-glucoses, the ratio of three kinds of rotamers around the C5-C6 bond could be calculated as *gg* : *gt* : *tg* = *ca.* 60 : 40 : 0, while for D-galactoses the ratio of the three rotamers was *ca.* 15 : 60 : 25. There, it could be first proved that the *tg*-conformation was negligible for D-glucoses and the *gt*-conformation was preferred by D-galactoses in water although D-galactoses had long been believed to prefer the *tg*-conformation.

This approach based on the combination of chiral deuteration and NMR

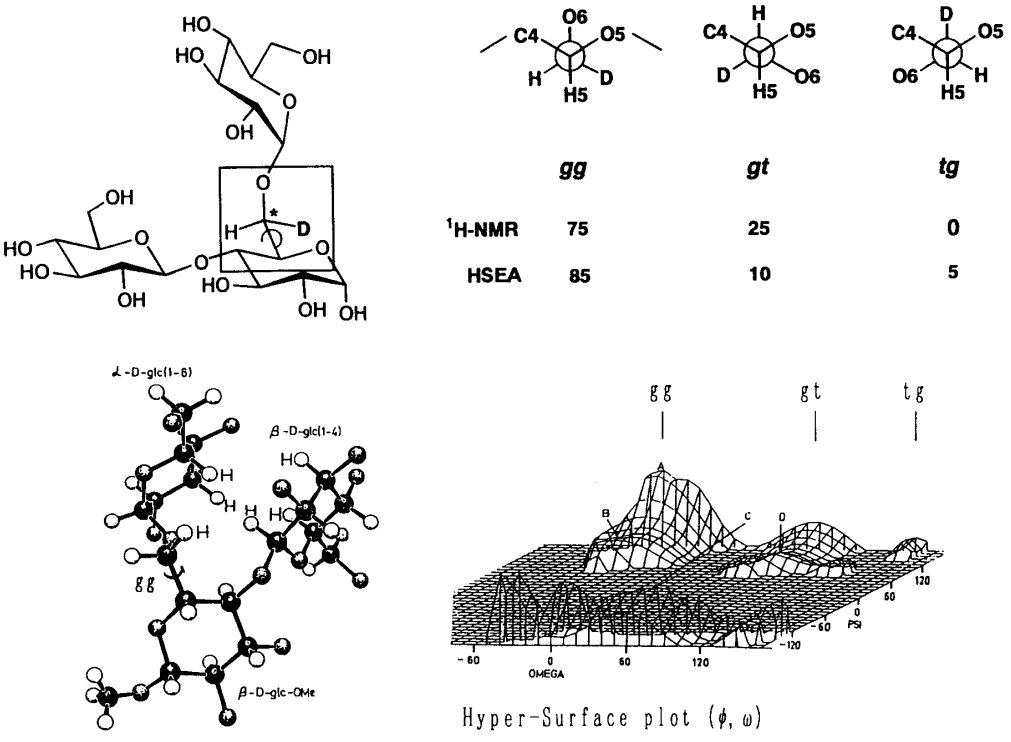


FIG. 5. Definition of three kinds of rotamers, *gg*, *gt*, nd *tg* around the C5-C6 bond of 1-6 linked saccharides and the comparion of the rotameric distributions calculated from ^{1}H-NMR method with HSEA energy calculations (*lit.* 23). The illustrated branching trisaccharide showed a exceptionally high *gg*-preference due to the hydrophobic interaction between 04 and 06 D-glucose residues.

spectroscopy was extended to a series of 1-6 linked di- and trisaccharides as the models of branching oligosaccharides. Some of di- and trisaccharides chirally deuterated at the C-6 position were summarized in Figure 3 and as follows; 1-6 linked glucoses and galactoses (20, Figures 2-4), di- and trisaccharides of a mucin type (21) and of a high mannose type (22), 4, 6-branching triglucoses (23) and N-acetylneuraminyl D-glucoses and D-galactoses (24). The conformational preferences around the C5-C6 bond at the 1-6 linkage point could be determined by measuring the vicinal coupling constants of H-6proR and H-6proS and by applying our equations in the same manner as the analysis of monosaccharides. Moreover, the linkage angles (ϕ, φ) were postulated from the chemical shifts of the two H-6 protons, NOE between H-1′ and H-6proR or H-6proS and deuterium effects on the relaxation times (22). The analyses of 1-6 linked disaccharides revealed that the conformational preference around the C5-C6 position showed a similar tendency to that of the corresponding monosaccharides in water solution.

For the conformational analysis of branching trisaccharides (four stereoisomers) HSEA calculation processed on GESA program (2) was also conducted, and the results were compared with the analysis based on chiral deuteration and NMR spectroscopy (Figure 5). These two results showed a similar tendency to each other though some deviation of the rotamer distribution around the C5-C6 was observed (23). The studies indicated that the conformation was largely affected in the presence of a sugar residue at 0-4 probably due to the steric interaction between the two sugars as 04 and 06. In the four stereoisomers, Glcβ1-4 (Glcα1-6) Glcβ0Me showed exceptionally high *gg*-preference probably due to the favored H-H hydrophobic interaction between the 04 and 06 residues to stabilize the conformer (Figure 5).

In conclusion, we have clarified the conformational property of the hydroxymethyl groups and the 1-6 linkage points of a series of mono- and oligosaccharides in water based on chiral deuteration and NMR spectroscopy. This approach and the results have beed widely applied in the analyses of higher oligosaccharides and in relating organic molecules by the other groups (25-34).

(ii) Highly Sensitive Analysis to Determine the D, L-Configurations Based on Fluorescent Chiral Derivatization and HPLC

Determination of sugar components in oligosaccharides or natural products is an inevitable problem not only in terms of their biochemical studies but also for stereochemical study. For the identification of sugar components, HPLC has been most widely employed instead of gas liquid chromatography (GLC) because of the former's ease of handling. The drawback of the HPLC method for sugar analysis is the lack of UV chromophore in sugar molecules. Therefore, for the detection of sugars, UV chromophore must be introduced into sugars before or after the HPLC separation. For the more sensitive HPLC analysis, a variety of

post-column or pre-column fluorescence labeling methods have been developed and applied for the sequence analyses of oligosaccharide chains (35–37).

On the other hand, sugars are optically active compounds bearing more than two asymmetric centers. This means that sugars have a pair of enantiomers, *i.e.*, D- and L-isomers, For the D, L-analysis of sugars, several gas chromatographic methods have been proposed (38–41) along with classical methods based on optical rotation or more sensitive ORD (42). However, chiral discrimination between D- and L-sugars has rarely been performed by HPLC. In natural compounds, unique sugars like L-glucosamine in streptomycin and L-galactose in marine products have been identified. Even in glycolipids or glycoproteins unusual sugars with L-configuration may be possible when they originate in special sources. Therefore, developments of simple and highly sensitive HPLC methods to discriminate sugar enantiomers will contribute much especially in the chemistry of natural products. In this study, we tried to develop a general HPLC method to determine the D, L-sugar components of oligosaccharides and natural products.

In order not only to separate enantiomeric sugars on the HPLC column but also detect them in a highly sensitive manner, application of a fluorescent chiral derivatizing agent will provide the most effective method. However, there was no fluorescent chiral agent developed for the analysis of sugars. In our separate study on the stereochemistry of glycerols and amino acids, we designed a new type of fluorescent chiral agent, (S)-TBMB carboxylic acid [(S)-2-*tert*-butyl-2-methyl-1, 3-benzodioxole-4-carboxylic acid, Figure 6] (43–45). With this agent, the esters and the amides show strong fluorescence [Ex. 310 nm (max), Em. 380 nm (max)] which can be applied for the highly sensitive HPLC analysis. The absolute configuration of this agent was determined by the X-ray crystallographic analysis as the L-phenylalanie derivative (46). Through the extensive application of this reagent for the D, L-analyses of amino acids (43–46), acylglycerides

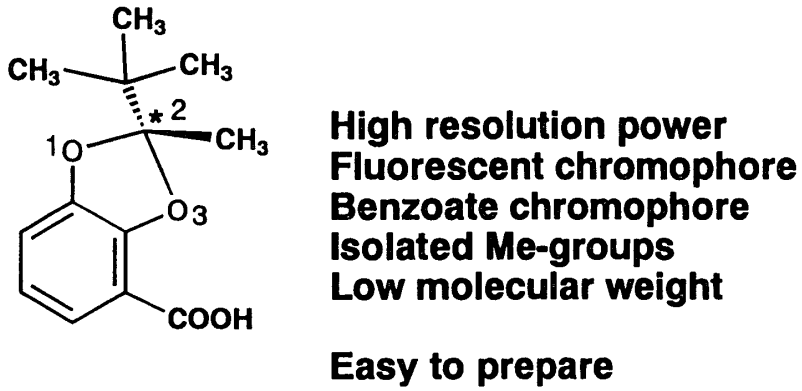
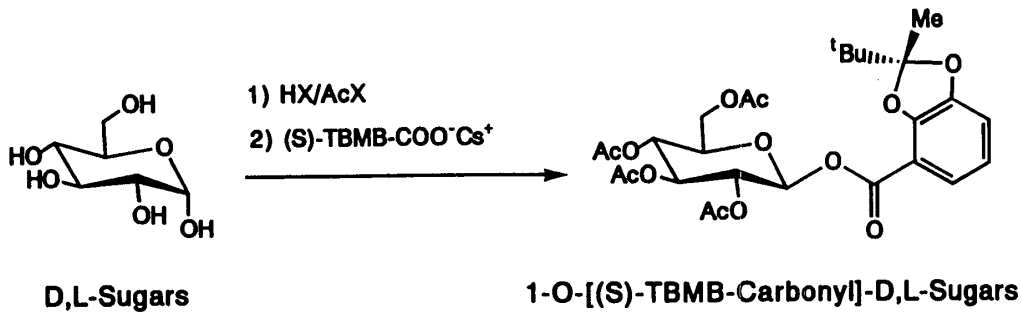


FIG. 6. The structure of (S)-(+)-TBMB carboxylic acid and its versatile functions as a chiral derivatizing agent.

(47-50) and the other chiral amines and alcohols, this agent has shown constantly high resolution power in the HPLC analysis.

Application of this agent for sugar analysis, however, could not be performed in the same manner as the analyses of amino acids and glycerides since sugars have many OH-groups reactive to this agent. In order to circumvent this problem, an alternative derivatization pathway was designed, which involved the activation of reducing sugars in the form of 1-halogenides (51-53, Figure 7). The reaction of 1-halogenides (X = Br or Cl) with (S)-TBMB carboxylic acid in a basic media gave 1-(S)-TBMB carboxylates in high yields (51). For glucose and galactose with an *equatorial* C2-OH group, this reaction gave a single compound with 1, 2-*trans* configuration, while for mannose and rhamnose with an *axial* C2-OH, it gave a mixture of 1, 2-*trans* and *cis* derivatives. This suggested the two types of the reaction mechanism; one through the S_N2 substituent and the other through the S_N1 substituent affected by neighboring participation of the C2-OAc group.

After the derivatization with (S)-TBMB carboxylic acid in this way, the enantiomeric D- and L-sugars could be well separated on reversed-phase HPLC column (ODS coulmn, CH_3CN : H_2O : *Iso*PrOH) and discriminated in a highly sensitive manner (0.1 pico mole amount of the column) by fluorometric detection. Moreover, the elution order was found to be governed by the absolute configuration at the C-1 position; (1S)-isomer was always eluted faster than the diastereomeric (1R)-isomer (Figure 8). This rule is useful to assign the D, L-



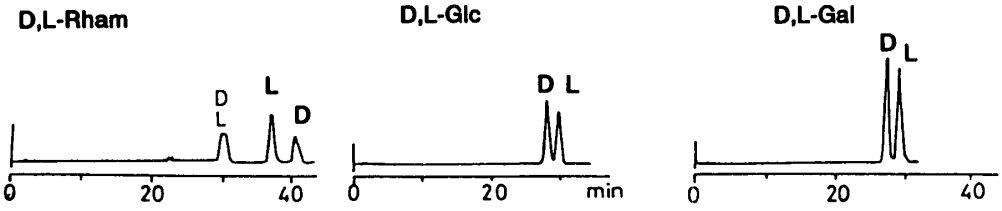


FIG. 7. Derivatization of reducing sugars with (S)-TBMB carboxylic acid and the HPLC separation of D, L-enantiomers. (ODS column, CH_3CN : H_2O : *Iso*-ProOH, *lit.* 52, 53).

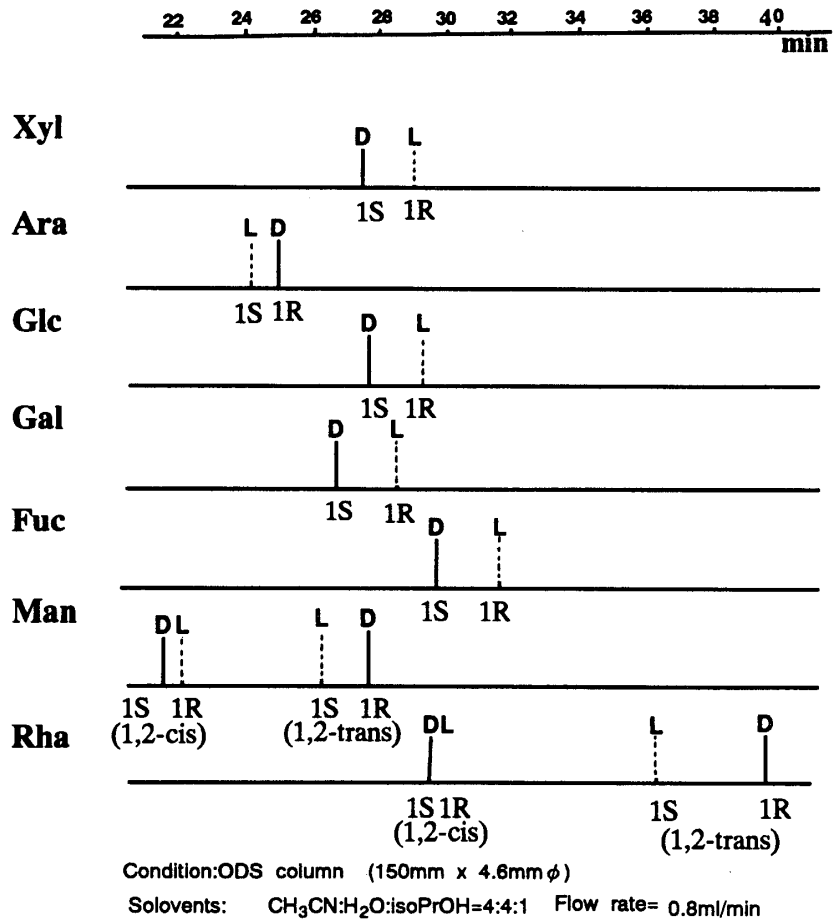


FIG. 8. Summary of HPLC separation of D, L-isomers derivatized with (S)-TBMB carboxylic acid (*lit.* 52, 53).
(1S)-Isomer was eluted always faster than the (1R)-isomer.

configuration of the parent sugars since the stereo chemistry of the 1-(S)-TBMB carboxylation could be well established as described above.

This approach could be successfully applied to the analysis of the D, L-sugar components of rutin (Figure 9) and kanamycin (Figure 10) (53). The HPLC charts in Figures 9 and 10 indicated that each of the sugar components was separated well enough to determine their D, L-configurations. Moreover, the sugar aglycons did not appear on the HPLC charts since the derivatization with (S)-TBMB carboxylic acid occurs selectively regarding reducing sugars.

In conclusion, a highly sensitive HPLC method to determine the D, L-sugar components of oligosaccharides and the other natural products has been established. In our independent study (46–50), this agent has been successfully applied to develop highly sensitive HPLC methods for the D, L-analysis of amino acids and glycerides. Thus, we have shown that (S)-TBMB carboxylic acid can be applied to the D, L-analyses of all of the three main biomolecules, sugars, amino acids and glycerides. (S)-TBMB carboxylic acid was designed as a versa-

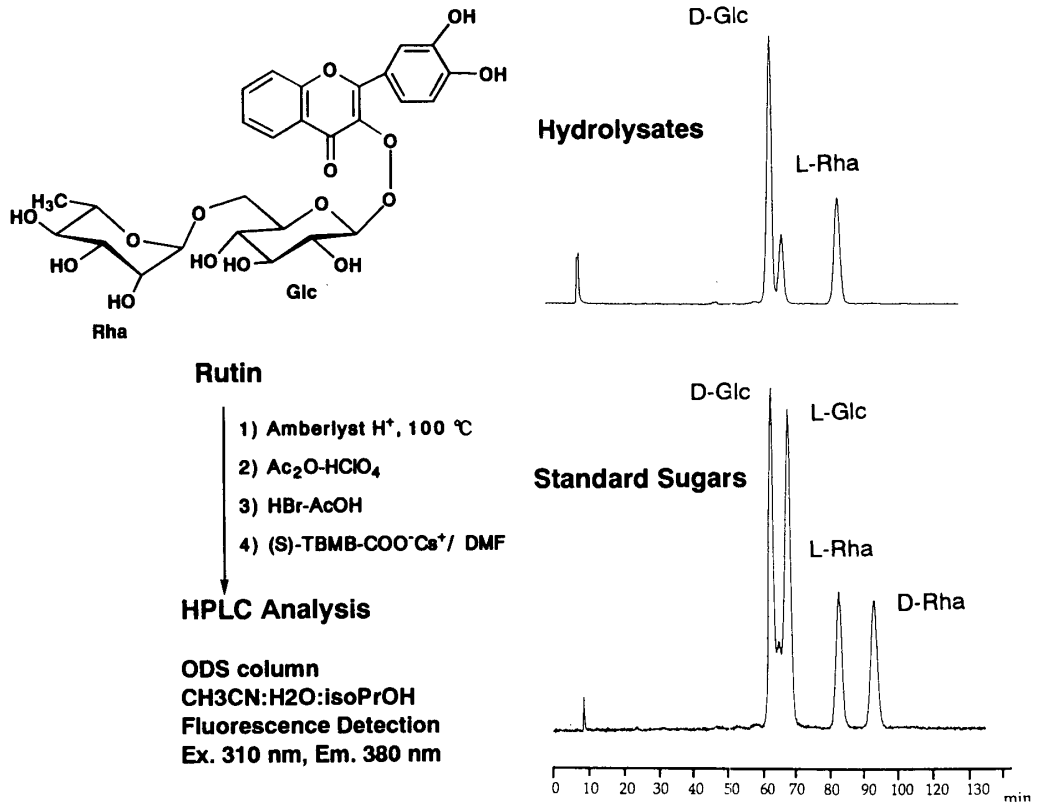


FIG. 9. HPLC analysis of D, L-sugar components in rutin.

tile reagent suitable not solely for the HPLC analysis. The agent has two isolated Me groups suitable for the NMR analysis, benzoate chromophore for the CD analysis and low molecular weight for the GLC analysis (Figure 6). Developments of alternative methods to analyze asymmetric compounds based on these functions are in progress along with the development of the HPLC method for the sequence analysis of oligosaccharide chains.

(iii) Discovery of The First Regiomistaken Reaction of UDP-Galactosyl Transferase

Since most of enzymatic reactions proceed in a stereoselective manner, enzymes have been effectively applied for synthetic purposes. Also for the syntheses of oligosaccharide chains or the modifications of sugar molecules, the use of enzymatic methods has collected large interest mainly because the reactions usually do not require multistep protections and deprotections nor hazardous metals like Ag or Hg neccessary for the chemical methods. The enzymes commonly used for the synthetic purpose are hexosyltransferases (like UDP-galactosyltransferase and CMP-sialyltransferase) and hexosidases (like β-glucosidases and β-galactosidases). Lipases have also been widely employed for the regioselective acylation or deacylation of sugars. As the extension of our

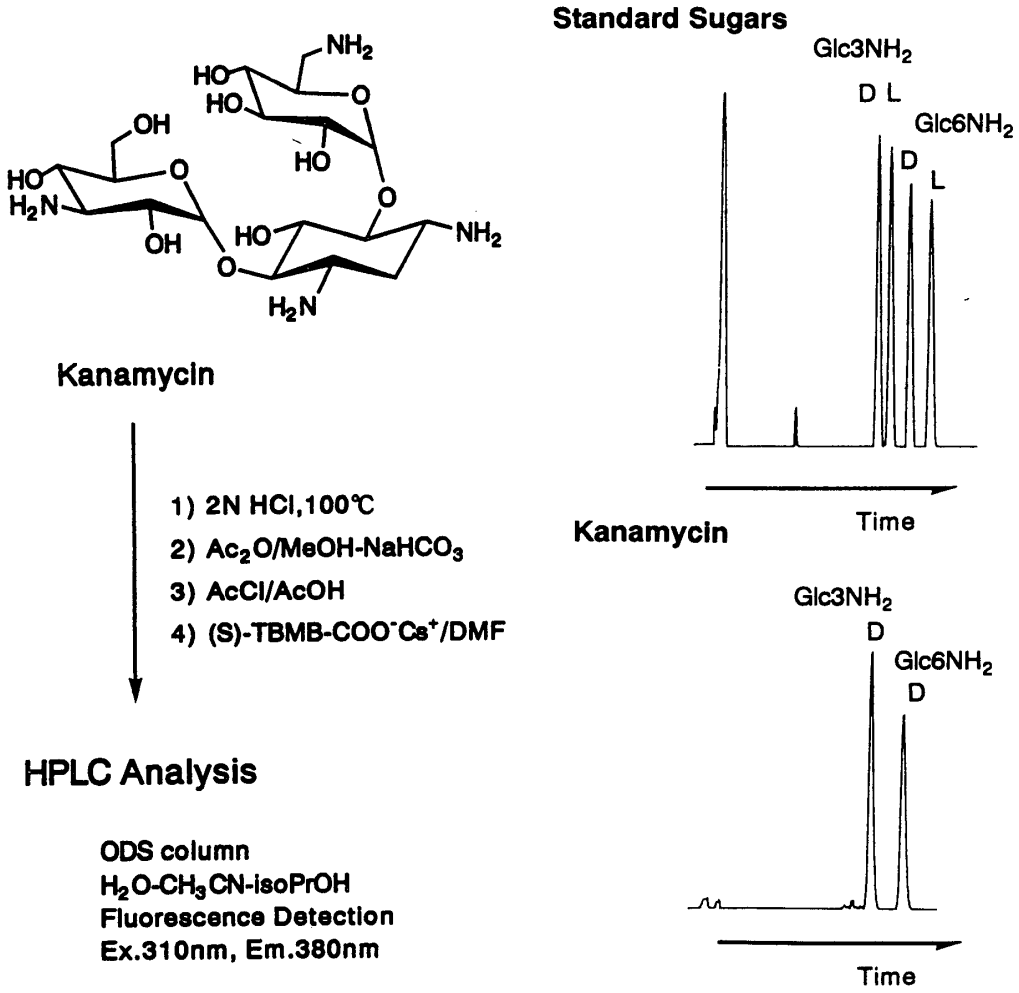


FIG. 10. HPLC analysis of D, L-sugar components in kanamycin (*lit.* 53).

stereochemical studes on carbohydrate molecules, we have investigated the stereochemistry of enzymatic reactions of sugars and the related organic molecules. In this section, stereochemistries of galactose oxidase reactions and then lipase reactions will be briefly described. At the end, the stereochemistry of UDP-galactosyltransferase reaction will be described in more detail.

Galactose oxidase reactions: The first target in our study was to clarify the stereochemistry of the galactose oxidase reaction (18). This enzyme oxidizes a hydroxymethyl group of D-galactose into an aldehyde probabaly *via* the selective oxidation of one of H-6proR and H-6proS. Here, we tried to clarify the selectivity by using chirally deuterated D-galactoses, (6R)- and (6S)-(6-$^2H_1$)-D-galactoses (15) which had already been prepared for the conformational analysis (16). The kinetic studies of the enzymatic reaction taking a deuterium effect into account (unpublished data) showed that more than 95% of H-6proS was selectively released during this oxidation. This selectivity was also interpreteted by

introducing a new enzyme-substrate interaction at the active site based on the *gt*-preference of D-galactose in water (see preceeding section, 16, 18, 20).

Lipase hydrolysis reactions: The next target was the lipase reactions of triacylglycerols (55-59). To our surprise, this hydrolysis had long been believed to proceed in a non-enantioselective manner; it was reported that lipases yielded racemic diacylglycerols from achiral triacylglycerols. This concept showed a clear contrast to the other lipase reactions giving optically active compounds from achiral substrates. Therefore, we assumed that the non-stereoselectivity in the lipase reaction of triacylglycerols should be attributed to the poor sensitivity of the analytical methods or to the racemization of products during the enzymatic reactions. Supported by this assumption, we started to develop a highly sensitive method to determine the D, L-configurations of diacylglycerols.

First of all, three dimensional structures of triacylglycerols were investigated by using chirally deuterated triacylglycerols (55, 56) and ^{1}H-NMR in the same way as for the sugar analysis in the preceeding section. The study indicated that the vicinal C-*O*-Acyl moiety always took the *gt*-preference over the *gg* and *tg* conformers, and this result led us to develop a spectroscopic method using circular dichroism (CD) to determine the absolute configuration of diacylglycerols and related acyclic alcohols (57). The CD method based on exciton chirality theory was applied to the analysis of the lipase reaction and enabled us to prove that some of lipases produced optically active diacylglycerols from achiral triacylglycerols (58, 59). For more sensitive analysis of diacyglycerols and monoacylglycerols, HPLC methods based on the derivatization with (S)-TBMB carboxylic acid have also been developed (47-50).

UDP-Galactosyltransferase Reactions (60-64): UDP-Galactosyltransferase (GalT) from bovine milk catalyzes the transfer of β-galactose from UDP-galactose to the 0-4 position of D-glucose in the presence of lactalbumin. In the absence of lactalbumin, the substrate affinity with *N*-acetyl-D-glucosamine is increased to afford *N*-acetyllactosamine (Figure 11). The stereo selectivity of this transfer reaction has been extensively investigated (65-68) to indicate that this enzymic reaction shows absolute regioselectivity at the 0-4 position of D-glucose and *N*-acetyl-D-glucosamine. Owing to its regioselectivity, GalT has widely been employed for the syntheses of di- and oligosaccharides bearing a lactose or *N*-acetyllactosamine unit at the non-reducing terminal unit (69-72).

In our separate interests in the substrate specificity of galactose recognition lectins (73-75), the modification of C-3 position (glucose unit) of lactose was expected to change the affinity with lectins since the C3-OH is involved in the center of the hydrogen bonding network. Besides the chemical synthetic pathways towards C-3 modified lactoses (76), we attemped to use GalT to prepare a variety of C3-modified lactoses starting from C-3 modified glucoses. Soon after we started this enzymatic approach, Wong *et al.* reported nearly the same works

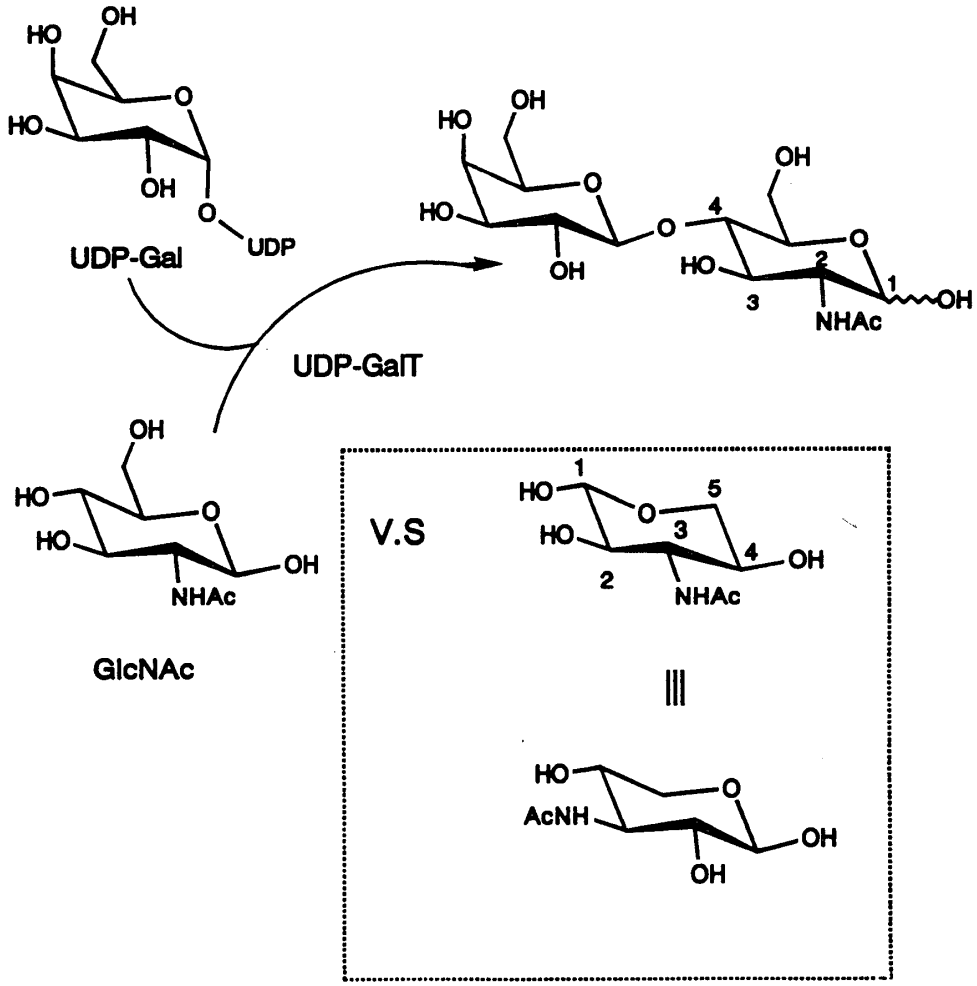
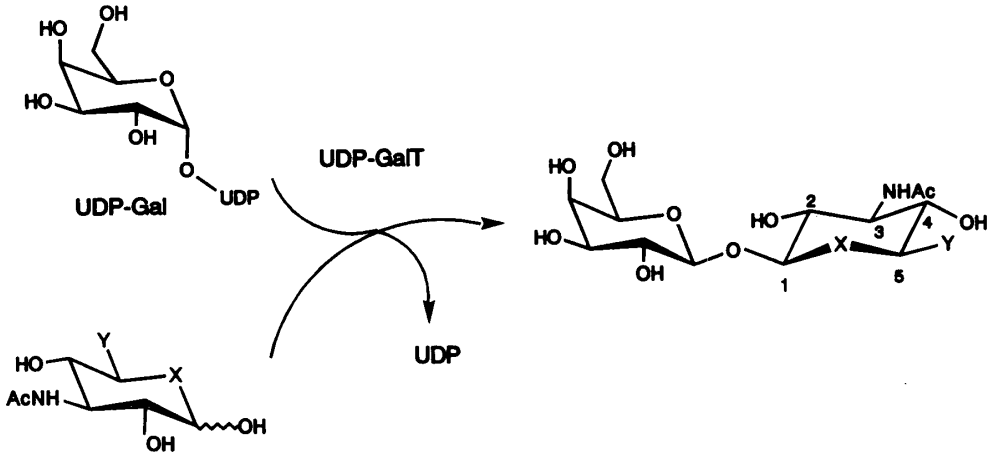


FIG. 11. Enzymatic synthesis of N-acetyllactosamie by UDP-galactosyl transferase (GalT) and the design of a new substrate Xyl3NAc which is expected to show an abnormal β Gall, 1-transfer reaction.

regarding the enzymic preparations of some C-3 modified lactoses and N-aceetyl lactosamines (77). Therefore, our attempts to prepare C3 modified lactoses with GalT were given up. This negative situation, however, resulted in helping us to find a new type of GalT reaction as described below (60-64).

Although the perfect regioselectivity of GalT is very useful for the enzymatic syntheses, this character has limited the use of this enzyme for the other purposes. Here, we wondered if GalT could transfer β-galactose to the OH-group rather than the C4-OH position though such a regiomistaken reaction had never been found. During the re-examination of the reported GalT reactions and its substrate specificities (65, 70), we noticed that β OH-1 may have a potential to become the reaction site since the stereo chemistry at 04 and 01 of β-D-glucose is identical to each other along the C1-C2-C3-C4 bonds. Thus, it was expected that the GalT could transfer galactose even to the anomeric position if the normal reaction at 0-



	X	Y
Glc3NAc	O	CH_2OH
Xyl3NAc	O	H
5-thio-Xyl3NAc	S	H

FIG. 12. A new type of GalT reaction producing β Gall, 1 linked disaccharides of β-trehalose type (*lit.* 60–64).

4 could be suppressed and the reaction at β OH-1 could be activated by chemical modifications. It was also suggested that GalT might have a pocket to accept an *N*-acetyl group, and the role of lactalbumin for the biosynthetic switching of lactose and *N*-acetyllactosamine seemed to be interpreted by the conformational changes of this pocket (65). This suggestion prompted us to introduce an *N*-acetyl group to the C-3 position of D-glucose (Glc3NAc) and D-xylose (Xyl3NAC) in order not only to deactivate the reaction at 0-4 but also to increase the reactivity of the β OH-1 groups (Figure 11).

As expected, the GalT reactions of Glc3NAc and Xyl3NAc occurred perfectly at the anomeric position to afford a disaccharide with a Gal β1, 1-linkage of β-trehalose type (62, 63). Thus, the first regio-mistaken reaction could be discovered in the GalT reaction (Figure 12). The same abnormal reaction could be also observed in the reaction of 5-thio Xyl3NAc where the ring oxygen -O- was replaced with -S- (62). Thus, a new 5-thio-disaccharide of β-trehalose type could be prepared for the first time by applying this abnormal GalT reaction.

Another interesting reaction was observed in the reaction of D-xylose; the GalT reaction of D-xylose gave a mixture of 1, 1-linked and 1, 4-linked disaccharides (63). This result first revealed that all of the GalT reactions do not proceed in a regioselective manner although GalT had shown absolute regioselectivity of either β Gall, 4 or β Gall, 1-transfer. Since D-xylose is widely distributed in nature, this result strongly implies the existence of 1, 1-linked disaccharides of β-trehalose type in nature by the action of GalT.

Consequently, these works could revise a common concept that GalT reaction proceeds regioselectively at the 0-4 position of D-hexoses. The results, therefore, have extended the potential of GalT for the oligosaccharide syntheses and given new insight into the substrate specificity of GalT (60).

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